Evidence from Rat Liver Nuclear Preparations that Latency of Microsomal UDP-Glucuronosyltransferase is associated with Vesiculation

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1. Nuclear, nuclear-envelope and microsomal preparations were prepared from rat liver, and their purity and morphology monitored by electron microscopy. 2. UDP-glucuronosyltransferase activity in microsomal preparations, but not in standard nuclear or nuclear-envelope preparations, displays latency from the criterion of being enhanced ('activated') by a range of detergents or the endogenous activator UDP-*N*-acetylglucosamine. 3. Nuclear preparations resemble activated rather than native microsomal preparations in failing to transfer glucuronic acid from 4-nitrophenyl glucuronide to 2-aminophenol. 4. Electron microscopy indicates that membranes of nuclear preparations and of our standard nuclear-envelope preparations remain, as *in vivo*, in a cisternal arrangement, whereas those of microsomal preparations are vesiculated. 5. In nuclear-envelope preparations in which vesiculation has been encouraged, the transferase can be activated by detergents. 6. We suggest that latency of UDP-glucuronosyltransferase results from vesiculation of membranes during preparation and that the latency of the microsomal transferase is largely a preparative artefact.

UDP-glucuronosyltransferase (EC 2.4.1.17) is well established as a membrane-bound microsomal enzyme which exhibits a marked degree of latency *in vitro* and which can be activated by a variety of treatments (see Dutton & Burchell, 1977). The relationship between such transferase activities assessed *in vitro* and those occurring in the intact cell therefore depends on the extent to which the features of latency observed *in vitro* exist *in vivo*. Transferase activity, measured in perfused liver, liver slices and isolated hepatocytes has often been considered to resemble that of latent rather than activated microsomal preparations *in vitro* (see Dutton & Burchell, 1977). However, unknown compartmentation of substrates, products, effectors and inhibitors of the enzyme may invalidate such comparisons. There is a marked morphological difference between the cisternal form of the endoplasmic reticulum of the living hepatocyte and the vesicular microsomal preparation produced from this organelle on disruption of the cell and used for

studies *in vitro*. Our approach to the problem of relating activities *in vitro* to those *in vivo* was to study UDP-glucuronosyltransferase activity in a subcellular fraction which has biochemical characteristics similar to those of the endoplasmic reticulum but largely retains its morphology *in vivo* on subcellular fractionation, i.e. the nuclear envelope.

We previously found that UDP-glucuronosyltransferase in liver nuclear preparations possesses specific activities (phospholipid-based) severalfold higher than those of microsomal preparations when neither has been subjected to known activating procedures (Fry & Wishart, 1976; Wishart & Fry, 1977). In the present work we show that this difference arises from different activation characteristics of the enzyme, only the microsomal transferase displaying latency. On comparing the transferase characteristics with the electron-microscopic morphology of our standard cisternal, nuclear-envelope and nuclear preparations, of largely vesiculated nuclear-envelope preparations and of vesicular microsomal preparations we have found that latency of UDP-glucuronosyltransferase is associated with vesicular rather than cisternal membrane preparations. A brief note of some of this work has previously been made (Wishart & Fry, 1978).

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Materials and Methods

Nuclear and nuclear-envelope fractions were prepared by the modification of the method of Kay *et al.* (1972), described previously (Fry & Wishart, 1976). The nuclear fractions were routinely checked by phase-contrast and electron microscopy for purity. Morphometry of the nuclear preparations [by the procedures described by Weibel & Bolender (1972), with five blocks and 20 photographs per preparation, a final magnification of 21 500 and a 2cm-line, 2cm-interval sampling grid (total line length 168cm) to determine membrane surface densities] indicated that less than 5% of the membrane present was not clearly associated with chromatin. Vesicular nuclear-envelope preparations were made by disrupting nuclei either mechanically with a Teflon/glass homogenizer in the absence of external Mg^{2+} or by mixing them with a large volume of 0.2M- $MgCl_2$ in 0.25M-sucrose solution. Unbroken nuclei were removed by a single centrifugation at 700g for 15 min, and nuclear-membrane vesicles were obtained from the supernatant by centrifuging at 105 000g for 30 min. The microsomal fraction was prepared by centrifuging the 700g supernatant of the 20% (w/v) liver homogenate in 0.25M-sucrose (buffered to pH 7.4 with 2mM- $NaHCO_3$) at 9000g for 20 min and then centrifuging the resultant supernatant at 105 000g for 30 min. All fractions were resuspended in 0.25M-sucrose (buffered to pH 7.4 with $NaHCO_3$)/1mM- $MgCl_2$. UDP-glucuronosyltransferase activity towards 2-aminophenol was assayed as before (Wishart & Fry, 1977) and activity towards 1-naphthol determined by the method of Otani *et al.* (1976). Transglucuronidation and the reverse UDP-glucuronosyltransferase reaction were performed as described by Berry & Hallinan (1974). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and phospholipid was measured after extraction (Albrink, 1959) and $HClO_4$ digestion of samples, by using a sensitive method for phosphate determination (Post & Sen, 1967).

For electron microscopy samples of the preparations suspended in 0.25M-sucrose/ $NaHCO_3$ /1mM- $MgCl_2$ were mixed with 20 vol. of 4% (v/v) glutaraldehyde in 0.1M-cacodylate buffer, pH 7.4. After 2h the fixed material was pelleted, post-fixed in 4% (w/v) OsO_4 , prestained by immersion in 0.5% (w/v) uranyl acetate overnight, dehydrated and embedded in Spurr's resin.

Results

The effect of Triton X-100 on UDP-glucuronosyltransferase activity towards 2-aminophenol in nuclear, nuclear-envelope and microsomal preparations is shown in Fig. 1. Clearly, over a wide

range of detergent concentrations, transferase activity in the nuclear and nuclear-envelope preparations is not significantly activatable, whereas in the microsomal preparation the transferase is activated to twice the activity of native preparations. In five experiments with 2-aminophenol as substrate the ratio of peak activity with Triton X-100 to activity with no detergent was 1.93 ± 0.30 (mean $\pm$ S.E.M.) for microsomal preparations, 1.12 ± 0.06 for nuclei and 1.06 ± 0.06 for nuclear envelopes.

Fig. 2 compares the effect of Triton X-100, other detergents and UDP-*N*-acetylglucosamine on transferase activity towards 2-aminophenol in preparations from the same homogenate. All treatments, except for that with digitonin, activated the microsomal transferase to about three times the activity of native preparations, the nuclear transferase remaining unresponsive. Digitonin was exceptional in that it produced some rise in nuclear transferase activity, confirming our previous findings (Wishart & Fry, 1977). However, its activation of the microsomal enzyme was double that of the other detergents, suggesting an 'extra' stimulating property of digitonin. Transferase activity in microsomal preparations exposed to the same high-density sucrose solutions as nuclei are during their preparation remained latent and activatable. So it seems unlikely that preparative procedures were responsible for activating any latent nuclear transferase.

Fig. 1. Comparison of the effects of different concentrations of Triton X-100 on UDP-glucuronosyltransferase activity towards 2-aminophenol in preparations of microsomes (Ms), nuclei (Nuc) and nuclear envelopes (NE) from adult male rat liver. For details see the text.

Fig. 2. Comparison of the effects of various concentrations of Triton X-100 (a), Lubrol 12 A9 (b), deoxycholate (c), digitonin (d) and UDP-N-acetylglucosamine (e) on UDP-glucuronosyltransferase activity towards 2-aminophenol in nuclear (▲) and microsomal (●) preparations from adult male rat liver

For details see the text.

In the preparation shown in Fig. 2, native nuclear and microsomal specific activities were respectively 130 ± 16 and 32 ± 2 nmol of 2-aminophenyl glucuronide formed/h per mg of phospholipid (mean $\pm$ S.E.M. for five duplicate assays). Micro-

somal specific activities, fully activated by Triton X-100, Lubrol 12 A9, deoxycholate, digitonin or UDP-N-acetylglucosamine, were respectively 89, 78, 124, 218 or 88 nmol of 2-aminophenyl glucuronide/h per mg of phospholipid. Native nuclear specific

activities were therefore closer to values for fully activated than for native microsomal preparations.

A further indication that the nuclear fractions were fully activated was that, like activated microsomal preparations, they did not transglucuronidate from 4-nitrophenyl glucuronide to 2-aminophenol. Without activator the ratio of 4-nitrophenyl glucuronide-dependent to UDP-glucuronic acid-dependent formation of 2-aminophenyl glucuronide for microsomal preparations was 0.12 ± 0.01 (mean $\pm$ s.e.m., $n = 4$) and for nuclei the value was 0.0058 ± 0.0026 ($n = 4$). In these experiments production of 4-nitrophenol from 4-nitrophenyl glucuronide occurred in the nuclear preparations at $42 \pm 6.5\%$ ($n = 3$) of the microsomal rate, and was therefore not rate-limiting.

Electron microscopy shows that the nuclear preparations used retain the nuclear membranes as a single layer or a double envelope around the chromatin, with the perinuclear cisternae open at several points to the extranuclear fluid (Plate 1a). The nuclear envelopes obtained by our standard procedure also maintain their cisternal character (Plate 1b), whereas the microsomal preparations consist of mixed vesicles (Plate 1c). Latency is therefore only seen in a preparation where access to the cisternal aspect of the membrane might be restricted.

The property of latency could be given to nuclear preparations by deliberately vesiculating the membranes (see the Materials and Methods section). In preparations showing a degree of vesiculation comparable with that in the microsomal preparations (as judged by electron microscopy; see Plates 1c and 1d) transferase activity to 1-naphthol was activatable by Triton X-100 or deoxycholate to $234 \pm 29\%$ ($n = 4$) of the activity in the absence of detergent, and the activity of unactivated preparations was only 81% of that of the parent nuclei. In this series transferase activity to 1-naphthol was activated by the factor 4.72 ± 0.76 (mean $\pm$ s.e.m., $n = 7$) in the microsomal preparations and by the factor 1.14 ± 0.12 ($n = 4$) in the nuclei. Microsomal preparations subjected to the procedure used to vesiculate the nuclear membranes retained a transferase activity and a degree of activatability not significantly different from those of the standard microsomal preparations.

With both 2-aminophenol and 1-naphthol as substrates, transferase specific activity (per mg of phospholipid) was significantly higher in nuclei than in fully activated microsomal preparations. Peak activity to 2-aminophenol was 122 ± 22 ($n = 5$) and 270 ± 54 ($n = 5$) nmol of glucuronide produced/h per mg of phospholipid for Triton X-100-activated microsomal preparations and nuclei respectively, and the corresponding values for 1-naphthol were 1788 ± 390 ($n = 7$) and 3486 ± 528 ($n = 7$). The

high native nuclear activity (compared with the microsomal) thus appears to be due to a combination of absence of latency and higher specific activity. The specific activities (phospholipid-based) of cisternal nuclear envelopes did not differ significantly from those of the nuclei, but those of the fully activated vesicular nuclear membranes were 1.64 ± 0.21 ($n = 4$) times those of the nuclei, and it seems likely that the vesicles leave behind on the chromatin more of the envelope phospholipid than of the envelope transferase (see Fry, 1976).

Discussion

The finding that rat liver UDP-glucuronosyltransferase activities in nuclear and cisternal nuclear-envelope preparations are not, as are those of the vesicular microsomal preparations and the largely vesicular nuclear-envelope preparations, activated by detergents or the endogenous activator UDP-*N*-acetylglucosamine suggests a possible correlation between membrane vesiculation and latency of the transferase. Transferase activities in nuclear and cisternal nuclear-envelope preparations resemble those of fully activated, rather than latent, microsomal preparations both in the activity (per mg of phospholipid) and in their inability to transfer glucuronic acid from 4-nitrophenyl glucuronide to 2-aminophenol ('transglucuronidation'). Berry & Hallinan (1976) have suggested that activation of the transferase occurs by facilitating access of UDP-glucuronic acid through the microsomal membrane to the enzyme on the luminal side of the microsomal vesicle, and that 'transglucuronidation' relies on UDP-glucuronic acid being trapped in the lumen of the intact microsomal vesicle. Such a compartmentational model of the microsomal transferase would appear a more attractive explanation of our present findings than one in which membrane vesiculation imposes the conformational changes which other workers have considered responsible for transferase latency (Zakim & Vessey, 1976). A parallel situation exists with glucose 6-phosphatase, another membrane-bound enzyme, which has been shown to exhibit latency in microsomal, but not in nuclear, preparations (Gunderson & Nordlie, 1973). In this case histochemical work shows that product is released to the vesicle or cistern lumen (Leskes *et al.*, 1971), so that at least part of the enzyme can be placed on the luminal side of the microsomal vesicles. That either access of substrate or cofactor, or release of product, occurs on the luminal side appears a necessary condition for any compartmentation theory of latency, but as yet there is no clear evidence that it is satisfied in the case of UDP-glucuronosyltransferase. Nilsson *et al.* (1978) studied the extent to which diazobenzenesulphonate (mol.wt. 186) could penetrate to (and inhibit) three microsomal enzymes, glucose 6-phosphatase, man-

EXPLANATION OF PLATE 1

Representative electron micrographs of (a) the purified nuclear fraction, (b) the standard nuclear-envelope preparation, (c) the microsomal fraction and (d) the vesiculated nuclear-envelope preparation

Magnifications: (a) $\times 3800$; (b)–(d) $\times 35000$. Nuclei (a) show a double-layered envelope with the perinuclear cisternae open to the medium at the occasional breaks in the outer membrane layer. The standard nuclear-envelope preparations (b) retain the double-layered envelope character seen in the nuclei [pore complexes (arrowed) are recognizable in (b) cut in longitudinal and transverse section]. In contrast, in (d) the envelope membranes have broken up into vesicles similar in size to the microsomal ones (c). For preparative details see the Materials and Methods section. All micrographs are from thin sections stained with lead citrate and uranyl acetate.

nose 6-phosphatase and UDP-glucuronosyltransferase, and also compared the effects of protein digestion on these three. Some of the results are in keeping with a luminal position for the transferase, but several differences between the effects on the phosphatases (considered to be lumenally sited) and the transferase led Nilsson *et al.* (1978) to suggest that the (latent) transferase might be placed on the cytoplasmic side of the membrane, but covered by a surface layer. This, like our own findings, could be compatible with either compartmental or conformational views of latency.

Both compartmental and conformational theories must therefore be considered when deciding which of our preparations *in vitro* best simulates the situation *in vivo*. As shown by the electron microscope, the morphology of the nuclear envelope in our nuclear preparations comes close to that of the intact cell, and it seems unlikely that the conformation of the nuclear transferase is significantly changed on isolation, whereas the structural pattern of the endoplasmic reticulum is completely broken down on homogenization and some change in physical constraint seems inevitable. If it is the conformation that the transferase takes up in vesicles that is responsible for latency, then the lack of latency in the cisternal (nuclear and envelope) preparations argues against its occurrence *in vivo*. If, however, latency is due to compartmentation, the important question is the degree to which access to or from microsomal sites is restricted. In the formation of microsomal vesicles from endoplasmic reticulum, access to luminal transferase sites becomes more restricted than *in vivo* in two ways: (1) the luminal fluid is broken up into small discrete units, each able to exchange only with the medium and across a bounding membrane, and (2) the surface area available for a given volume of luminal fluid to exchange across is substantially decreased. *In vivo* the cisternal fluid may be considered a continuum, with each section able to equilibrate within milliseconds with its neighbour. Local depletion owing to uneven transferase distribution or activity would be minimal, and all transferase sites would share in the ease of access to the cytosol conferred by even a few areas of higher permeability in a way not possible in microsomal preparations. The transformation into vesicles is also restrictive if permeability and transferase density are uniform. Stereological analysis of whole liver has indicated a surface-area/volume ratio for endoplasmic reticulum of $88\mu\text{m}^{-1}$ (Weibel *et al.*, 1969), whereas a similar analysis of our microsomal vesicles gives a ratio of only $28\mu\text{m}^{-1}$. So if permeability is a limiting factor, the geometric change on isolation, irrespective of other effects, would more than halve the amount of substrate or product passing to or from luminal sites, and this could account for much of the latency that

we observed. On this basis the nuclear and envelope preparations would represent more closely the condition in the cell, even though the gaps in the outer membranes take the higher-regional-permeability situation to an extreme not found *in vivo*.

By associating latency with vesiculation, our results therefore provide grounds for questioning the existence of latency *in vivo*, whether it is due to compartmentation or conformation. We suggest that isolated nuclei may retain the features of the transferase *in vivo* better than do microsomal preparations, and that the latency of the microsomal transferase is mainly an artefact, produced when the cisternal endoplasmic reticulum vesiculates into 'microsomes' on cell disruption.

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